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Effects of Vanadium Ions in Different Oxidation States on Myosin ATPase Extracted from the Solitary Ascidian, *Halocynthia roretzi* (Drasche)

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ABSTRACT—Some ascidians are known to accumulate vanadium ion within their tissues by 10^6-fold as that in sea water and store the metal ion in its reduced tetravalent and/or trivalent states. It is also well known that phosphoenzymes are inhibited by pentavalent vanadium ion over a range of 10 nM to 1 mM. In the present experiment we have therefore examined the effects of vanadium ions in different oxidation states on the activity of myosin ATPase extracted from the mantle of the ascidian, *Halocynthia roretzi*, in order to know the mechanism for protectin phosphoenzymes against inhibition by the massive amounts of vanadium ion within their tissues. The activity of myosin ATPase was inhibited by pentavalent vanadium ion but was not inhibited by tetravalent or trivalent vanadium ion. The addition of 5 mM ascorbic acid, which is known to reduce pentavalent vanadium ion to tetravalent state, to the reaction mixture reduced the inhibitory effect of pentavalent vanadium ion on the ATPase. Based on the results, one of the reasons that the ATPase within the ascidian is not inhibited *in vivo*, in spite of the high level of vanadium within ascidian tissues, may be that the pentavalent vanadium ion which is accumulated from sea water is readily reduced to the tetravalent and/or trivalent oxidation states by the endogenous reducing substances in ascidian tissues.

INTRODUCTION

Since it has been demonstrated that a physiological concentration of pentavalent vanadium ions causes inhibition of Na^+-K^+-ATPase [1, 2], the effects of vanadium ions on various enzymes have been studied by many investigators [cf. 3, 4]. Ascidians are known to accumulate very high levels of vanadium within their tissues [5–10], at concentrations that are, in some cases, much higher than those that are able to inhibit the activity of phosphoenzymes [4]. When actomyosin ATPase, extracted from several species of ascidians, was allowed to react with pentavalent vanadium ions *in vitro*, it was found that the vanadium ions, at concentrations of 10 μM to 100 μM, caused an apparent inhibition of ATPase activity [11].

Ascidians must, however, have an efficient mechanism for protecting phosphoenzymes against inhibition by the massive amounts of vanadium ions within their tissues, but there are no reports of such a mechanism.

In this study, we have examined the effects of vanadium ions in different oxidation states on the activity of myosin ATPase, extracted from the ascidian mantle, having chosen this enzyme as representative of ascidian phosphoenzymes.

MATERIALS AND METHODS

Halocynthia roretzi (Stolidobranchia) were purchased from a fisherman at Asamushi, Aomori, Japan. Myosin was prepared from the mantle by the method of Obinata *et al.* [12].

Each reaction mixture (1 ml) contained 20 mM

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Tris-maleic acid buffer (pH 8.0), 120 mM KCl, 10 mM $CaCl_2$, 0.5 mM ADP, and myosin (200 μg protein/ml reaction mixture). Vanadium ions were added to the reaction mixture by dilution from concentrated stock solutions which were prepared just before use. The mixture was preincubated without ATP for 15 min at 25°C, then the reaction was started by addition of a solution of ATP (final concentration of ATP was 1 mM). The reaction was terminated by the addition of 1 ml of ice-cold 20% trichloroacetic acid. All assay were carried out in triplicate. Liberated inorganic phosphate (Pi) was measured by the method of Allen [13]. Protein was determined, using Coomasie brilliant blue, with bovine serum albumin as standard [14].

To examine the effects of vanadium ions in different oxidation states on the myosin ATPase, the following compounds were tested; Na_3VO_4(V), $VOSO_4$(IV), and VCl_3(III). Experiments were also carried out in the presence of 5 mM ascorbic acid, as a reducing agent. This agent was added to the reaction mixture at the same time as the pentavalent vandium compound was added.

RESULTS

The specific activity of myosin ATPase, obtained from the mantle of the ascidian, was about 1.89 μmoles Pi/mg protein/min. This activity tended to decrease gradually with storage.

The degree of inhibition of ATPase activity by vanadium compounds was expressed as the percent of the initial control value which corre-

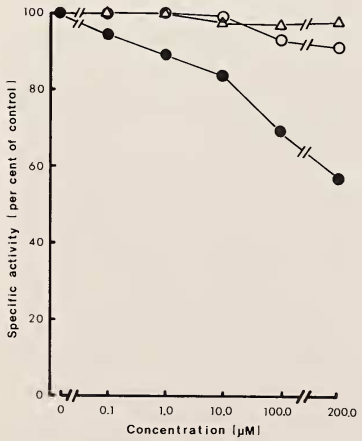

FIGURE. 1. Effects of vanadium ions in different oxidation states on myosin ATPase extracted from *Halocynthia roretzi*. ●: $Na_3VO_4(V^{+5})$; ○: $VOSO_4(V^{+4})$; △: $VCl_3(V^{+3})$. The data points shown are the averages of triplicate determinations. The initial control value of 100% corresponds to liberation of 1.89 μ moles Pi/mg protein/min.

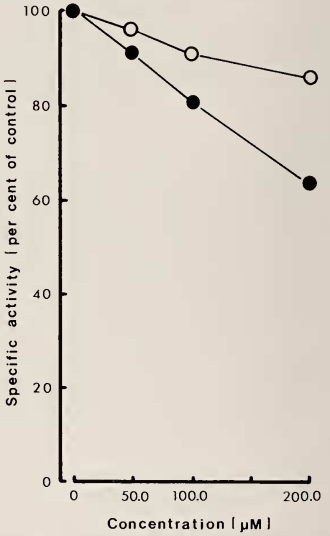

FIGURE. 2. Diminution of the inhibitory effect of pentavalent vanadium ions by ascorbic acid. ●: Na_3VO_4; ○: Na_3VO_4+5 mM Ascorbic acid. 5 mM ascorbic acid was added to the reaction mixture at the same time as the pentavalent vanadium compound was added. The data points shown are the averages of triplicate determinations. The initial control value of 100% corresponds to liberation of 0.54 μ moles Pi/mg protein/min.

sponded to the liberation of 1.89 μmoles Pi/mg protein/min, as shown in Figure 1. The results clearly demonstrated that pentavalent vanadium ions, at a concentration of 100 μm, are a powerful inhibitor of the myosin ATPase extracted from the ascidian mantle. The half-maximum inhibition of activity was seen at approximately 200 μM vanadium ion(V). In contrast to this result, tetravalent and trivalent vanadium ions, in the same range of concentrations, had little or no inhibitory effect on the enzymatic activity. Maximum values of only 8.2% and 2.6% inhibition were observed when 200 μM tetravalent and trivalent vanadium ions, respectively, were included in the reaction mixture.

Ascorbic acid is known to reduce pentavalent vanadium ions to the tetravalent state under physiological conditions [15]. Therefore, we examined whether the addition of ascorbic acid to the reaction mixture could diminish the inhibitory effect of pentavalent vanadium ions on the enzymatic activity. Figure 2 shows that 5 mM ascorbic acid can clearly diminish the inhibitory effect of pentavalent vanadium ions. Two hundreds μM pentavalent ion reduced the activity of myosin ATPase by about 37% in this experiment, while in the presence of 5 mM ascorbic acid, the same concentration of pentavalent vanadium ions reduced the activity by only 14.8%.

DISCUSSION

In spite of a massive accumulation of vanadium within ascidian tissues [5–10], at concentrations that are thousands to million times higher than those that can inhibit ATPases [1, 2], ascidians are able to protect their enzymes from the inhibitory effects of vanadium. The present results demonstrate that the activity of the ascidian myosin ATPase is inhibited only by pentavalent vanadium ions, and is not inhibited by tetravalent and trivalent vanadium ions (Fig. 1). In other words, the enzyme are not subject to the inhibition by vanadium so long as the oxidation state of vanadium ions is not pentavalent even so ascidians contain high levels of vanadium ions within their tissues. It has been known, in fact, that the oxidation state of the vanadium ions that are stored within ascidian tissues is tetravalent and/or trivalent [5, 9, 16–22] although the vanadium ions dissolved in sea water is in the pentavalent state [23].

Ascorbic acid [24, 25], glutathione [4, 26], cysteine [27], and NADH [28], which are common to cells, have been known to be able to reduce pentavalent vanadium ions to tetravalent vanadium ions *in vitro,* which suggests that these reducing agents are involved in oxidation-reduction reactions of vanadium ions *in vivo.* It is, therefore, probable that endogenous reducing agents such as the ascorbic acid in the ascidian tissues change the oxidation state of the vanadium ions and keep vanadium ions in reduced states. The presence of such reduced vanadium ions would explain why ascidian phosphoenzymes are not subject to the inihibirory effects of the vanadium within their tissues.

Although free tetravalent vanadium ions are oxidized to pentavalent vanadium ions within a few minutes in aqueous solution at neutral pH, the tetravalent ions are stable in combination with various cellular components [29]. In fact, the results obtained from the present experiments show a clear difference between the effect of pentavalent vanadium ions and that of tetravalent vanadium ions on the activity of myosin ATPase (Fig. 1), and imply that the tetravalent vanadium ions are also stabilized in the present assay system. The partial inhibitory effect exerted by the tetravalent vanadium ions may depend on the partial oxidation of these ions to pentavalent vanadium ions in the reaction mixture.

Furthermore, it is known that trivalent vanadium ions are appreciably hydrolyzed below pH 2.2 but above that pH they dimerize and precipitate. Thus, it is probable that a water-soluble species does not exist under neutral and basic conditions in a simple aqueous solution [30]. However, the actual chemical forms of vanadium ions in complex solutions, such as the reaction mixture described in MATERIALS AND METHODS, are little understood. Since the trivalent vanadium ions have no inhibitory effect on the activity of the ATPase, it is unlikely that the trivalent vanadium ions are quickly oxidized to the tetravalent and/or pentavalent form. However, if the trivalent vanadium ions dimerize and precipitate immediately after addition to the medium, this

result can be explained. There is also another possibility: the trivalent vanadium ions may be able to maintain their oxidation state by complexing with some molecules in the reaction mixture. In such case, the trivalent vanadium ions themselves would have no effect on the enzymatic activity. The actual behaviour of trivalent vanadium ions in the reaction mixture is, however, unknown at the present time.

As described above, ascidians contain the vanadium ions in tetravalent and/or trivalent state [5, 9, 16–22]. We have isolated a vanadium-binding substance (Vanadobin) from the blood cells of *Ascidia sydneiensis samea,* which substance can maintain the vanadium ions in reducing form of tetravalent state both under aerobic and anaerobic conditions [21]. However, the corresponding substance to keep the vanadium ions in trivalent state in ascidian tissues is not yet extracted.

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